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What determines growth potential and juvenile quality of farmed fish species?

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Abstract

Enhanced production of high quality and healthy fry is a key target for a successful and competitive expansion of the aquaculture industry. Although large quantities of fish larvae are produced, survival rates are often low or highly variable and growth potential is in most cases not fully exploited, indicating significant gaps in our knowledge concerning optimal nutritional and culture conditions. Understanding the mechanisms that control early development and muscle growth are critical for the identification of time windows in development that introduce growth variation, and improve the viability and quality of juveniles. This literature review of the current state of knowledge aims to provide a framework for a better understanding of fish skeletal muscle ontogeny, and its impact on larval and juvenile quality as broadly defined. It focuses on fundamental biological knowledge relevant to larval phenotype and quality and, in particular, on the factors affecting the development of skeletal muscle. It also discusses the available methodologies to assess growth and larvae/juvenile quality, identifies gaps in knowledge and suggests future research directions. The focus is primarily on the major farmed non-salmonid fish species in Europe that include gilthead sea bream, European sea bass, turbot, Atlantic cod, Senegalese sole and Atlantic halibut.

Key words: aquaculture, fish growth, methodology to assess growth, myogenesis, protein accretion, skeletal muscle ontogeny.

Introduction

Enhanced production of high quality and healthy fry is a key target for a successful and competitive expansion of the aquaculture industry. Although large quantities of fish larvae are produced, survival rates are often low or highly variable and growth potential is in most cases not fully exploited (Conceição *et al.* 2010). The larvae survival rates for the major Mediterranean farmed species of marine fish is commonly around 10% (personal communication, various Mediterranean hatchery managers), indicating significant gaps in our knowledge concerning optimal nutritional and culture conditions. Under these circumstances the aquaculture environment is likely to impose strong selection on the species concerned with profound consequences for their domestication. In addition, culture conditions themselves exert a potent epigenetic influence on embryonic development, particularly environmental temperature and nutrition. There is increasing evidence that early events imprint an individual physiological memory resulting in long-term effects on postnatal growth and physiological function, both in animals and humans (Rehfeldt *et al.* 2011). The environment determines the rate of myogenesis, the composition of sub-cellular organelles, patterns of gene expression, the number and size of muscle fibres (reviewed by Johnston 2006) and influences protein turnover and the

efficiency of protein deposition (Conceição *et al.* 2008). In farmed fish these factors have persistent effects on economically important traits such as growth performance, body composition and flesh quality in subsequent life history stages. The consequences and implications of such epigenetic processes for aquaculture production is underappreciated and an important goal for future research. Understanding the mechanisms that control early development and muscle growth are critical for the identification of time windows in development that introduce growth variation. The aim is to develop strategies to intervene and influence future growth, whilst reducing the incidence of developmental disorders and their long-term consequences that have a negative impact on product quality. There is a clear need for an improvement of the scientific knowledge basis that will support sustainable growth of the European aquaculture industry by supplying high quality fish juveniles.

A larval fish research network LARVANET, COST Action FA0801 'Critical success factors for fish larval production in European Aquaculture: a multidisciplinary network', was established in 2008 and included researchers and producers working with fish larvae. The Action was intended to integrate knowledge obtained in national and European research projects, as well as practical experience, in order to improve the quality of fish larvae used in aquaculture. This literature review of the current state of knowledge aims to provide a framework for a better understanding of fish skeletal muscle ontogeny, and its impact on larval and juvenile viability and quality. Larvae quality is taken in its broad sense, and understood as fish larvae with outstanding survival and growth rates, with a minimum of skeletal deformities and other abnormalities, with a good growth potential during the juvenile stage, and with the ability to resist to environmental challenges during the whole life-cycle. This review focuses on fundamental biological knowledge relevant to larval phenotype and quality and, in particular, on factors that affect the development of skeletal muscle. It also discusses the available methodologies to assess growth and larvae/juvenile quality, identifies gaps in knowledge and suggests future research directions. The focus is primarily on the major farmed non-salmonid fish species in Europe that include gilthead sea bream, European sea bass, turbot, Atlantic cod, Senegalese sole and Atlantic halibut.

Development of skeletal muscle

Embryonic, larval and juvenile muscle growth: the origin and regulation of myogenic progenitor cell activity

Skeletal muscle derives from the somites formed from the paraxial mesoderm in a rostral to caudal progression, and represents 40–60% of fish body mass. Fish myotomes are composed of fast-twitch (white) and slow-twitch (red) fibres arranged in discrete layers and supported by anaerobic (phosphocreatine hydrolysis, glycolysis) and aerobic metabolic pathways, respectively. Muscle fibres with an intermediate aerobic capacity and contraction speed, but with a high potential for anaerobic glycolysis are found between fast and slow muscle layers in most species, and first appear in the larval or early juvenile stage (Rowlerson & Veggetti 2001). Larvae rely on cutaneous respiration prior to the development of the muscle capillary circulation and in active pelagic species both slow and fast muscle fibres have high volume densities of mitochondria, e.g. 46% and 26%, respectively, in Atlantic herring reared at 15°C (Vieira & Johnston 1992). The dependence of larvae on aerobic metabolism is correlated with high fatigue resistance and a rapid clearance of lactic acid following strenuous activity (Franklin *et al.* 1996). Larvae have much higher maximum tail-beat frequencies than juvenile stages and distinct patterns of sustained swimming activity, which is related to their small size and distinctive morphology (Blaxter 1988).

Muscle formation in teleost fish involves the production (hyperplasia, or recruitment) and subsequent enlargement of muscle fibres (hypertrophy and elongation). Myogenesis is common to all vertebrates and consists of serial complex events involving the specification, proliferation, differentiation, migration and fusion of precursor cells to form multinucleated muscle fibres (Fig. 1). In several teleost species, three distinct phases of fibre production can be recognized (reviewed by Steinbacher *et al.* 2006; Rescan 2008). The first phase is entirely embryonic in which adaxial and posterior somitic cells give rise to two morphologically and functionally distinct muscle types forming the primary myotome. A second phase that spans the late embryo and early larval stages involves the production of new muscle fibres in discrete zones (stratified hyperplasia). The third phase starts in larvae and continues into adult stages, and involves new fibre production throughout the myotome giving rise to a mosaic of fibre diameters (so-called mosaic hyperplasia). The relative timing and importance of each phase varies (Table 1) and may be related to the evolutionary history, growth potential and final body size attained by each species. In species of importance to aquaculture, mosaic hyperplasia is the main phase contributing to the growth of muscle (Johnston 2006).

Knowledge of the earliest events in myogenesis is largely derived from studies of the model species, *Danio rerio* (Brent & Tabin 2004; Devoto *et al.* 2006). Distinct lineages of muscle fibre types are specified prior to segmentation in the embryo and depend on inductive signals from adjacent tissues, such as the neural tube, the notochord, and the dorsal and lateral ectoderm (reviewed by Rescan 2008). Mononucleate adaxial cells adjacent to the notochord are

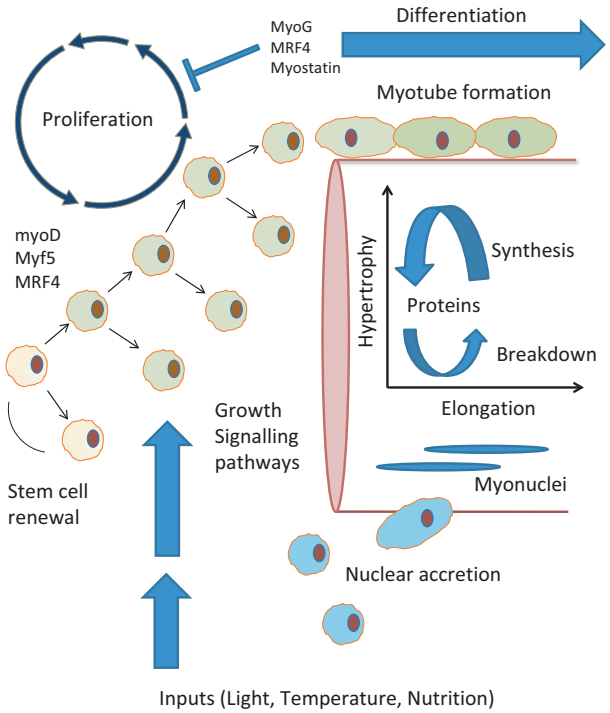

Figure 1 A model of muscle growth in teleost fish. The model assumes a rare stem cell population that can undergo an asymmetric division to produce a daughter cell that becomes committed to the myogenic lineage under the influence of myogenic regulatory factors (myoD, Myf, MRF4). These cells then undergo several rounds of proliferation to produce much more numerous myogenic progenitor cells (MPCs). Myogenin (MyoG), MRF4 and myostatin are part of a complex genetic network regulating the exit of MPCs from the cell cycle and the initiation of terminal differentiation involving the fusion of MPCs to form myotubes, myofibrillargenesis and sarcomere assembly. Inputs to these pathways (light, temperature, nutrition) determine the balance between proliferation and terminal differentiation and hence the production of MPCs required for growth. Other growth signalling pathways including IGF-mTor control protein synthesis and degradation determining the rate of fibre hypertrophy and elongation. As fibres increase in diameter and length additional MPCs are absorbed to maintain the nuclear to cytoplasmic ratio within certain limits.

fated to the slow muscle lineage, elongate and migrate through the somite to form a superficial monolayer of fibres under the influence of sonic hedgehog (SHh) secreted from the notochord. Myogenic differentiation involves four master transcription factors (myogenic regulatory factors, MRFs: myoD, mf5, myogenin and MRF4 (myf6)) plus MADS-box containing myocyte enhancing factor MEF2 proteins (Hinits *et al.* 2007). The MRFs act downstream of, or in parallel with, the paired domain and homeobox-containing transcription factors Pax 3 and Pax 7 (Fig. 2; Buckingham & Vincent 2009), with several Sox genes contributing to the control of muscle differentiation (Rescan & Ralliere 2010). The cells at the major horizontal septum retain a connection with the notochord and are recognized as muscle pioneer cells with a distinct programme of gene expression. SHh signalling induces the transcriptional regulator Blimp1 in slow muscle pioneer fibres, which functions as an epigenetic switch globally repressing fast-fibre specific genes and overcoming the actions of sox6, a repressor of slow-fibre-specific genes (Baxendale *et al.* 2004). Adaxial cells begin to differentiate while still in the segmental plate, and start expressing contractile proteins (myosin) of both slow and fast muscle fibres very early (Rescan *et al.* 2001). Shortly after their incorporation into somites they elongate and differentiate into slow muscle fibres forming a monolayer on the external surface of the embryonic myotome, underneath the dermomyotome. The anterior and posterior compartments of the early somite comprise two distinct cell populations with different fates (Stellabotte & Devoto 2007). Midway through segmentation the somite undergoes a rearrangement driven by a secreted cytokine-signalling pathway (Hollway *et al.* 2007) such that the anterior compartment becomes a layer external to the slow muscle layer (Fig. 2). This external cell layer that becomes distinct only after the formation of a primary myotome has been reported in several marine fish species (Mascarello *et al.* 1995; Lopez-Albors *et al.* 2003; Silva *et al.* 2008). Gene expression analysis supports a homology between amniote dermomyotome and teleost external cells (Devoto *et al.* 2006). In contrast, cells of the posterior somite differentiate into fast muscle fibres. Dermomyotome cells expressing Pax3/Pax7 contribute to dermal cells and to fin muscle precursors in anterior myotomes and myogenic progenitor cells (MPCs). It has been suggested that myotomal MPCs undergo asymmetric divisions and one of the daughter cells migrate through the slow muscle layer and elongate into the new fibres of the germinal zone to initiate the formation of the embryonic lateral medial fast muscle under the influence of fgf8 (fibroblast growth factor 8) signalling (Groves *et al.* 2005; Hollway *et al.* 2007). It has been shown that during the second phase of myogenesis, myogenic precursors detaching from the dermomyotome are a major source of myogenic cells driving stratified hyperplasia, at the dorsal and ventral extremes of the myotomes and also at the lateral boundaries of the fast muscle domains (Steinbacher *et al.* 2006; Hollway *et al.* 2007; Stellabotte *et al.* 2007). This myogenic capacity of the external cells has been demonstrated recently in zebrafish (Hollway *et al.* 2007; Stellabotte *et al.* 2007), pearlfish (Marschallinger *et al.* 2009) and trout (Dumont *et al.* 2008), but several other teleosts including sea bass, gilthead sea bream and blackspot sea bream retain a monolayer of undifferentiated cells on the external surface of the myotome into the early juvenile period (Veggetti *et al.* 1990; Mascarello *et al.* 1995; Lopez-Albors *et al.* 2003; Silva *et al.* 2008). Additional slow fibres form in the

Table 1 Onset of hyperplasia stages in different marine species

Species	Age/size of fish at the onset of hyperplasia		
	Stratified hyperplasia	Mosaic hyperplasia	Author
Dicentrarchus labrax	Start: 10 days; 0.5 cm (notochord flexion; first feeding) Up to: 41 days; 3.3 cm	Start: 27–80 days; 1–3.5 cm (notochord flexion; end met) Up to: still at 48 cm, 350 g; adult stages	Veggetti *et al.* (1990), Lopez-Albors *et al.* (2003) and Alami-Durante *et al.* (2007)
Dentex dentex	Start: 8–9 days (first feeding) Up to: 35 days; 1.33 cm	Start: 14 days; 0.49 cm Up to: Still by 90 days	Albors *et al.* (2010)
Gadus morhua	Start: 6 days; 0.45 cm (onset of first feeding) Up to: 30 days; ~0.74 cm (after onset of met)	Start: non specified Up to: still by 1 kg; finish by 100 cm	Greer-Walker (1970), Galloway *et al.* (1999a) and Johnston and Andersen (2008)
Hippoglossus hippoglossus	Start: 0.1 cm, 150 day Up to: onset of met	Start: non specified Up to: still by ~80 g	Galloway *et al.* (1999b) and Campinho *et al.* (2007)
Pagellus bogaraveo	Start: 5 days (first feeding) Up to: 23 days (end larval period)	Start: ~70 days (juvenile) Up to: still by 140 days	Silva *et al.* (2008, 2010)
Pagrus major	Start: 14–17 days; 0.35–0.7 cm (after first feeding; two layers of red fibres) Up to: 1.1 cm	Start: 2 cm Up to: non specified	Matsuoka and Iwai (1984)
Pleuronectes platessa	Start: 8 days (before met) Up to: still by 80 days; 0.12 cm (by the end met; still single red muscle)	Start: 10.4 cm (after met) Up to: adult stage; 26.2 cm	Brooks and Johnston (1993)
Salmo salar	Start: Before hatching Up to: first feeding	Start: first feeding Up to: 60–70 cm; adult seawater life	Johnston and McLay (1997)
Sparus aurata	Start: 8 days; 0.36 cm Up to: 20 days; 0.43 cm	Start: 60–90 days (post-met) Up to: still by 150 days	Mascarello *et al.* (1995) and Rowlerson *et al.* (1995)
Solea senegalensis	Start: pre-met; 8 days; 0.41 cm Up to: 30 days; ~1.3 cm	Start: post-met; 22 days; 0.85 cm Up to: 30 days; ~1.3 cm	Campos *et al.* (2012a)
Solea solea	Start: 21 days Up to: end of met	Start: ~2.5 months (end met) Up to: 1 year	Veggetti *et al.* (1999)
Scophthalmus maximus	Start: 11–26 days (by the onset of met; two layers of red muscle) Up to: non specified	Start: non specified Up to: non specified	Calvo and Johnston (1992) and Gibson and Johnston (1995)

Met, metamorphosis.

late embryo/early larval phase independently of SHh signalling, which is required for the formation of those embryonic slow muscle fibres from adaxial cells (Barresi *et al.* 2001).

Stratified hyperplasia has been identified widely in many species (Table 1) and is the major source of new fibres during late embryonic and early postembryonic growth. In most fish species, the appearance of intermediate fibres occurs close to the horizontal septum during the stratified hyperplastic growth phase (reviewed by Rowlerson & Veggetti 2001). By the early larval stage a resident population of Pax7 expressing myogenic precursor cells is evident throughout the myotome and these cells are thought to fuel the dramatic increase in muscle mass during ontogeny. In several marine species, the larval germinal zones are evident at around the onset of first swim and are generally depleted during metamorphosis or in the period shortly after (Galloway *et al.* 1999a,b; Campinho *et al.* 2007; Silva *et al.* 2010). The first attempts of the larvae at cruise swimming in search of food are extremely important for their survival at that age, but there is no trend in the timing of stratified hyperplasia (SH) in relation to the onset of exogenous feeding (Table 1): in gilthead sea bass, sea bream, red sea bream, halibut and cod hyperplastic growth seems to be triggered by endogenous energy sources, as a preparation for rapid growth after the onset of exogenous feeding (Rowlerson *et al.* 1995; Galloway *et al.* 1999a,b; Alami-Durante *et al.* 2006).

The basic module of embryonic embryogenesis involves myoblast specification, migration, elongation, fusion and terminal differentiation, and innervation and is recapitulated in larval, juvenile and adult stages (Fig. 1). Muscle

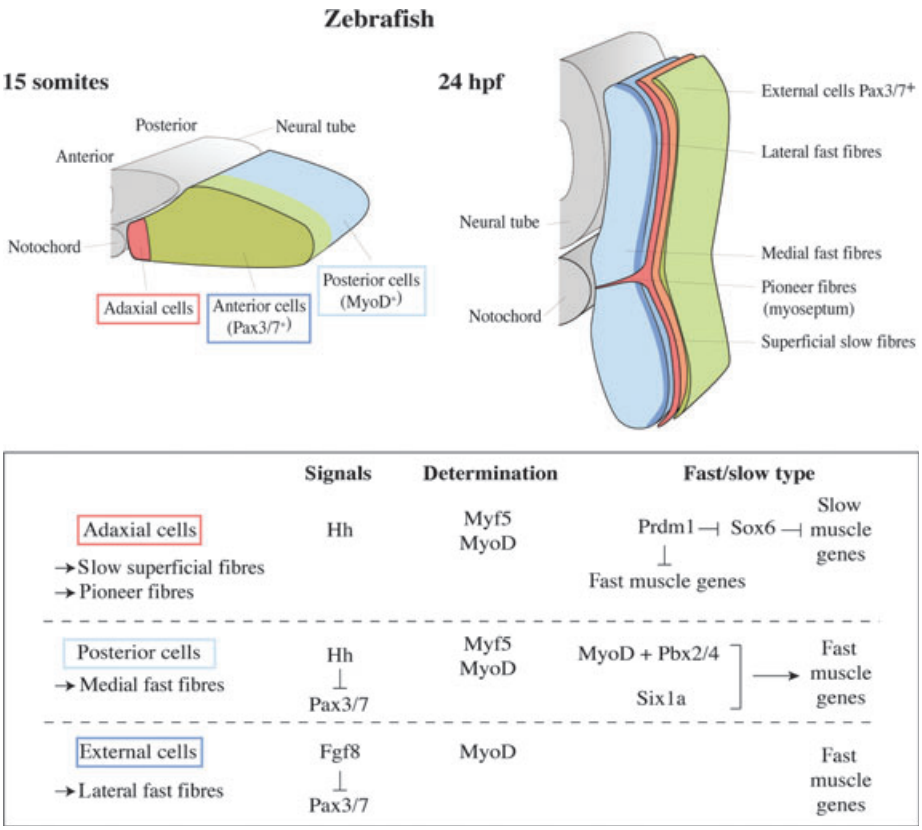

Figure 2 Slow and fast muscles segregate from the onset of myogenesis in the zebrafish embryo. Slow muscles are derived from the adaxial cells (in red). In the epithelial somite, anterior cells (green domain) express first Pax3 then Pax7, whereas posterior cells (blue domain) already express MyoD and will contribute to the medial fast fibres. During development, the somite undergoes a rearrangement and at 24 hours post fertilization, the Pax3/7 positive cells are now in a dermomyotome-like position. Adaxial cells differentiate into slow pioneer fibres that form the myoseptum and into slow fibres that migrate laterally across the medial fast fibres to form the most superficial layer of the myotome, the superficial slow fibres. After formation of the embryonic myotome, Pax7 positive cells colonize the myotome in order to form a second major wave of fast fibres (lateral fast fibres, dark blue region) and resident progenitor cells within the muscle. Adapted from Buckingham and Vincent (2009).

fibres attach via short tendons to the myosepta and along their lengths to other fibres. The expansion of muscle fibres with growth is accompanied by nuclear accretion to maintain the nuclear to cytoplasmic ratio within certain limits (Koumans *et al.* 1991; Johnston *et al.* 2003). Muscle is a mechanochemical transduction system and mechanical signalling plays an important, but poorly understood, role in growth regulation. A complex network of collagenous connective tissue is built around individual fibres and bundles of fibres by fibroblast cells forming connections with the myosepta and skeleton. The complexity of the genetic networks regulating hyperplasia and hypertrophy undoubtedly further increases during the larval and early juvenile stages as the neuroendocrine system, capillary and lymphatic circulations develop allowing new possibilities for signalling between tissues and with the external environment (Johnston *et al.* 2011).

The final and most important phase of hyperplasia involves the proliferation of myogenic precursor cells (MPC) present throughout the myotome that subsequently fuse to form myotubes on the scaffold of existing fibres to produce a typical mosaic appearance of fibre diameters, a process termed mosaic hyperplasia (Fig. 1). This process continues after the juvenile stage (Table 1), in contrast with mammals and birds where hyperplasia stops shortly after birth (Rehfeldt *et al.* 2011). The embryological origin of myogenic precursors for this mosaic hyperplasia phase is still unclear, but is probably the embryonic external cell layer (Devoto *et al.* 2006; Hollway *et al.* 2007). The growth rate and maximum size of a fish is strongly regulated by the intensity and duration of the mosaic hyperplastic growth. The largest and fastest growing fish generally show greater hyperplasia than slow growing fish (Weatherley *et al.* 1988; Kamler 2008). Moreover, the ability of teleosts to grow rap-

idly and to attain a large ultimate size is dependent on the body length at which recruitment of new muscle fibres into the growing axial muscle ceases. In seven species of freshwater fish the production of white muscle fibres was found to continue until around 40% of the maximum body length, after which growth was entirely by fibre expansion (Weatherley *et al.* 1988). Numerous genes have been implicated in myotube production including *Rac*, *Dock 1*, *Dock5*, *crk* and *crk-like* (Moore *et al.* 2007; Pajcini *et al.* 2008). Powell and Wright (2011) have shown recently that the zebrafish orthologues of a vertebrate-specific cell surface receptor pair, JAM-B and JAM-C receptors, are essential for fusion of myogenic progenitor cells to form syncytial muscle fibres. The myoblasts that contribute to new fibre production and nuclear accretion are likely to have distinct phenotypes (Fig. 1), but it is not known whether myoblast fate arises early in development or is specified later in response to local signalling.

Growth plasticity in fish species implies an adaptive responsiveness in the dynamics of myotomal muscle (favouring hyperplasia vs hypertrophy of existing fibres) to changing environmental factors such as temperature and food supply, and has considerable intra- and interspecific variation. Natural selection tends to maximize the scaling of exchange surface areas, and intracellular efficiency, by minimizing the scaling of transport distance and times (West *et al.* 1999). These constraints will probably limit the maximum diameter of muscle fibres that is around 150–200 μm in most fish species (Rowlerson *et al.* 1995; López-Albors *et al.* 2008). Johnston *et al.* (2012) proposed the 'optimum fibre size (OFS) hypothesis that predicts that selection tends to minimize the energetic costs of ionic homeostasis including those associated with the maintenance of the muscle membrane potential (approximately −70 mV)'. On theoretical grounds, as the fibre diameter increases the decrease in surface to volume ratio should reduce passive ion leakage across the sarcolemma and hence the requirement for ATP-dependent ion pumping. In the case of notothenioid fishes from Antarctica, low temperature (−1.9 to 2°C) reduces the metabolic rate and relaxes diffusional constraints permitting very large diameter white fibres (up to 650 μm) with low maintenance costs (Johnston *et al.* 2003). As a consequence, the lifetime production of white fibres in the icefish (*Chaenocephalus aceratus*) was <10% of that of another notothenioid, *Eleginops maclovinus*, from the Patagonian shelf which reaches a similar maximum body size (85 cm), but has evolved in a more temperate environment (Johnston *et al.* 2003). Interestingly, large reductions in fibre number in icefish species were also accompanied by a loss of the mosaic hyperplasia phase of growth (Johnston *et al.* 2003). Fibre size varies between populations exposed to different selective pressures, including those associated with domestication, and therefore has high relevance for aquaculture when considering the selection of broodstock. The rapidity and universal nature of fibre size optimization suggests that selection is occurring on standing allelic variation in a relatively limited number of myogenic genes (Johnston *et al.* 2012). The insulin-like growth factor (IGF) – mechanistic target of the rapamycin (mTor) signalling pathway – transduces environmental including nutritional signals to regulate protein turnover and myoblast proliferation/differentiation with growth (Erbay *et al.* 2003; Seiliez *et al.* 2008a). By manipulating inputs to this pathway in replicated dwarf and large-bodied arctic charr, evidence was obtained for adaptive modifications in several pathway genes, including mTor (Macqueen *et al.* 2011). Thus allelic variation (in the case of population variation) or *de novo* mutations (in the case of adaptive radiations) would seem promising candidates on which selection might act to affect fibre size optimization. Whatever, the precise molecular mechanism(s) the resultant changes in fibre size distribution have the potential to alter both energy allocation during growth as well as the texture and hence the eating quality of the flesh. Muscle is the final product in fish farming and consumers show a preference for a firm texture. White fibre number and size distribution are important determinants of the textural characteristics of fish flesh (Hurling *et al.* 1996; Johnston 1999; Periago *et al.* 2005). The maximum fibre number can vary between different genetic strains, and can be modulated by environmental conditions, with important implications for end-product quality (Johnston 1999). A significant correlation between fibre density and several textural parameters, including firmness, was reported in different fish species using instrumental methods (Periago *et al.* 2005) or a sensory panel (Hurling *et al.* 1996; Valente *et al.* 2011). Other factors contributing to flesh texture include pH, water content, lipid levels, the concentration of collagen sub-types and hydroxylysyl pyrdinoline crosslinks (Hagen *et al.* 2007). The selection of production practices able to maximize growth by fibre recruitment should result in flesh with a firmer texture.

Control of muscle mass

Somatic growth represents the balance between catabolic and anabolic components of protein metabolism (protein turnover; Fig. 1). Protein turnover is thought to have roles in the removal of defective proteins, and the supply of amino acids as substrates for energy production or as precursors to synthesize new enzymes or structural proteins (Conceição *et al.* 2008). In all organisms, the rates of protein synthesis and degradation in individual muscle fibres are carefully regulated. Indeed, even a small increase in synthesis or a small reduction in degradation if sustained over

time, may result in a marked accretion of muscle in the organism. In addition, protein turnover has been linked to metabolic plasticity of an organism, and a reduced protein turnover may lead to reduced plasticity in case of nutritional or environmental challenges (Kiørboe *et al.* 1987; Conceição *et al.* 2001). Protein turnover has been proposed as an indicator of metabolic plasticity because it is positively correlated with the speed at which an organism can respond to a change in environmental conditions (Conceição *et al.* 2001).

Protein deposition in muscle normally results from a coordinated increase in both protein synthesis and protein degradation (Houlihan *et al.* 1988, 1993; Reeds 1989). Therefore, any stimulation of muscle growth implies that the concomitant increase in protein synthesis must be enough to allow both the deposition of new protein and an increase in protein turnover. However, in fish larvae the rate of protein degradation does not always follow the rates of protein synthesis, and may even remain constant or be reduced (Houlihan *et al.* 1992; Conceição *et al.* 1997a,b). It has been proposed that fish larvae may decrease the rate of protein degradation or reduce the costs of protein synthesis, in order to respond to a strong selective pressure for high efficiency of protein deposition (Kiørboe *et al.* 1987; Conceição *et al.* 1997b).

Fish larvae, because of their very high growth potential, represent an excellent model to study the interactions between protein turnover, growth performance and viability. Engrola *et al.* (2009) observed that a sub-optimal feeding regime impairs larval fish protein utilization and growth performance in the short and long term. Immunostimulants present in the diet increase protein degradation without affecting growth performance of turbot larvae (Conceição *et al.* 2001), increasing larval viability and survival in the face of environmental or disease stress.

Protein synthesis

Protein synthesis in fish larvae seems to follow the general trends observed in adult fish and mammals (Houlihan *et al.* 1995b). Protein synthesis increases with growth rate, dietary protein level (Fauconneau *et al.* 1986b), temperature (Fauconneau *et al.* 1986a) and ration size (Fauconneau *et al.* 1986a,b; Houlihan *et al.* 1992).

As nutrition is a key regulator of protein accretion, through the modulation of the GH/IGF system activity, understanding the major regulators of protein homeostasis is paramount for improved protein retention. The central mediator nutrient sensing protein pathway is the PI3K/AKT/TOR (target of rapamycin; Erbay *et al.* 2003). When activated through feeding, it promotes mRNA translation and protein synthesis, resulting in the regulation of cell growth and proliferation, and cellular metabolism. This regulatory mechanism allows an organism to coordinate nutritional information to achieve balanced growth by regulating cell size and cell proliferation (Hietakangas & Cohen 2009). Zebrafish development was promoted in a concentration-dependent manner after IGF-1 stimulation by activating the phosphatidylinositol 3-kinase (PI3K) signalling pathway (Pozios *et al.* 2001). The mechanism is composed of two complexes (TORC1 and TORC2) that regulate growth in different ways. Target of rapamycin complex 1 (TORC1) responds to both growth factors and changes in local amino acid levels, therefore is responsible for the adjustment of protein synthesis rates. Studies in rats have demonstrated that an amino acid supplementation stimulates the TOR pathway in order to optimize the muscle anabolic response to each meal, however, does not increase the rates of protein synthesis (Norton *et al.* 2009). Activation of the TOR pathway in juvenile rainbow trout is regulated by feeding, and amino acids are essential for enhanced protein synthesis (Seiliez *et al.* 2008a, 2011; Lansard *et al.* 2009). Nevertheless the cluster of regulatory pathways of protein synthesis, protein degradation and energy sensing is not yet fully elucidated in vertebrates. Fish larvae with their tremendous growth potential may be a good model in this endeavour.

Protein degradation

Protein degradation is part of healthy muscle growth and metabolism. Muscle atrophy occurs when rates of protein degradation exceed the rates of protein synthesis. Protein degradation can be mediated by four main proteolytic systems: calpains, caspases, lysosomes and the ubiquitin-proteasome system (UPS; Jackman & Kandarian 2004). A review of proteolytic systems is beyond the scope of this article and so only brief comments are included on UPS, because it represents the basic cellular machinery for atrophy in mammalian muscle (Ciechanover 1998) and the calpain superfamily of proteases, because its members can be regulated by nutrition, are involved in myofibrillargenesis and are important in post-mortem ageing of fish flesh (Delbarre-Ladrat *et al.* 2004).

The ubiquitin-proteasome system plays a pivotal role in the degradation of short-lived and regulatory proteins important in a variety of basic cellular processes, including the rapid removal of proteins, regulation of gene transcription, functioning of the immune system, and as a source of amino acids (Lecker *et al.* 2006). Briefly, prior to degradation, a target protein undergoes a three-step process that covalently links a polyubiquitin chain to the substrate. Three enzyme components are involved in this process, E1 (Ub-activating enzyme), E2 (Ub-conjugating enzymes) and

the key enzymes that confer specificity to the system, E3 (ubiquitin protein ligase), that present substrate recognition sites. The ubiquitinated substrate can then be recognized and degraded by the 26S proteasome, resulting in peptides of 7–9 amino acid residues.

Bodine *et al.* (2001) identified a small subset of genes that were always upregulated in several atrophy models, two of which encoded ubiquitin ligases: Muscle RING Finger 1 (MuRF1), and a Muscle Atrophy F-box (MAFbx), also known as atrogin-1. In rainbow trout and salmon juveniles it was shown that fasting enhanced the expression of atrogin-1 (Seiliez *et al.* 2008b; Valente *et al.* 2012) and also the level of polyubiquitinated proteins in muscle (Seiliez *et al.* 2008b). Fasting in Atlantic halibut (Hagen *et al.* 2009), European sea bass (Terova *et al.* 2007) and gilthead sea bream (Montserrat *et al.* 2007) induced a downregulation of IGF 1 expression, usually restored to normal levels after re-feeding. Recently, it was shown in trout primary myocytes that leucine supplementation attenuates muscle degradation via minimizing gene expression of E3 ligases that may downregulate the UPS pathway (Cleveland & Weber 2010). These results suggest that protein synthesis and degradation pathways are similar between fish and mammals.

Calpains are a superfamily of Ca^{2+} regulated proteases involved in numerous physiological processes including cell death and apoptosis, cell motility, signal transduction, myoblast fusion and myofibrillargenesis (Goll *et al.* 2003). Calpains 1 and 2 are ubiquitously expressed in different tissues and are activated by micro- or millimolar- concentrations of Ca^{2+}, respectively, and with more than 100 substrates identified including cytoskeletal proteins, kinases, membrane receptors and transcription factors (Goll *et al.* 2003). During the spawning season sea bass white muscle showed an increase of calpains, suggesting a modulation of protein synthesis and/or protein degradation pathways to cope with a demanding physiological activity (Ladrat *et al.* 2000). Feed deprivation modulated calpains pathway in rainbow trout white muscle by concomitantly decreasing calpain transcript abundance and increasing calpastatin (specific inhibitor of calpains) transcript abundance (Cleveland *et al.* 2009). Calpain 3, often considered muscle-specific, was much more highly expressed in white muscle of Atlantic halibut than calpain 1, 2 or 11, and was also expressed at low levels in spleen and ovary (Macqueen *et al.* 2010). In halibut, calpain 1 was upregulated with fasting and downregulated with feeding, whereas calpain 1 and 11 were induced by feeding and calpain 2 was independent of nutritional status, indicating different roles for family members in regulating the balance between protein catabolism and growth in fish muscle (Macqueen *et al.* 2010).

Genetics of muscle growth

Comparative studies in teleosts suggest that in spite of the conservation of myogenic mechanisms some aspects of the regulation of gene expression during muscle ontogeny may be species-specific and related to particular environmental conditions or life-style (Hall *et al.* 2003; Campinho *et al.* 2007). Moreover, the whole genome duplication at the base of the teleost radiation, followed by additional duplication events in other lineages such as the salmonids, created multiple copies of genes involved in muscle growth pathways, increasing the potential flexibility of growth regulation in teleosts compared with mammals. The four myogenic factors (MyoD, MRF4, Myf5, myogenin) provide a good example. They are considered ancient paralogues generated during whole genome duplication in the lineage leading to vertebrates. Subsequently, teleost whole genome duplication, further polyploidization events and gene loss determined the number of paralogue genes in each fish species, which is particularly evident in teleost *MyoD* genes. Within Teleostei, Acanthopterygii have two paralogues, *MyoD1* and *MyoD2*, whereas Ostariophysi (zebrafish) have only *MyoD1* gene and salmonids expressed three *MyoD1* paralogues – *MyoD1a*, *MyoD1b*, *MyoD1c* – but no *MyoD2* (Macqueen & Johnston 2008). The different *MyoD* paralogues in salmonids and Acanthopterygii have sub-functionalized and they exhibit distinct expression patterns during development, yet together they recapitulate the expression pattern of the single *MyoD1* (Weinberg *et al.* 1996; Tan & Du 2002).

Modern aquaculture, based on an increasing array of recently domesticated fish species, has early embarked on the quest of favourable traits and their underlying gene networks. The first genes to be targeted were those of the somatotropic axis, genes of myogenic regulatory factors and transforming growth factors. The identification of polymorphisms in certain genes and association with growth traits in different species have suggested growth hormone (GH), insulin-like growth factors (IGFs) and myostatin (MSTN) as candidate genes for marker-assisted selection programmes (De-Santis & Jerry 2007; Wringe *et al.* 2010). Soon, the 'omics' explosion provided the tools for the development of large-scale genomic resources, facilitating the study of genomes and their interacting elementary structures (Canario *et al.* 2008). So far, the list of candidate genes has expanded to include genes associated with muscle fibre differentiation (*SMYD1*, *RTN1*, *HSP90A*), myoblast proliferation and cell cycle (*DRG1*, *CEBPD*), protein degradation pathways (*MuRF1*, *MAFbx*, *CTSL1*), muscle structural proteins (*TnC*, *TnT2*, *actin2*) as well as mitochondrial genes encoding for elements of the oxidative phosphorylation pathway (NADH dehydrogenase subunit

1, cytochrome b, ATPase 6; Bower & Johnston 2010; Salem *et al.* 2012).

Variations in DNA sequence are known to underlie trait variation. Either the gene polymorphisms themselves or other genes in linkage can be used as genetic markers to select fish with particular trait characteristics, called marker assisted selection (MAS). The regions of the genome that contribute to the shaping and variation of a given trait, including growth, are known as quantitative trait loci (QTL). The QTLs, once identified, can be used in the development of marker-assisted selection schemes to screen for selection candidates. Furthermore, QTLs offer the advantage that live fish can be screened for post-mortem traits (post slaughter fillet quality), or traits that affect survivorship (resistance in a challenge test) (Sonesson 2007). To date the identification of candidate genes and QTLs affecting growth related traits in marine fish species are scarce. A QTL located in LG1 in European sea bass was significantly associated with six morphometric traits, including the standard length and body length and depth (Rowlerson *et al.* 1997). In turbot, a significant QTL for sex determination was identified (Martinez *et al.* 2009) and a wide QTL analysis for body weight, length and Fulton's condition factor was performed (Martinez *et al.* 2000). In the latter study eight turbot families were screened and up to 11 significant QTLs were identified collectively for body weight, length and Fulton's condition factor. However, the high variation of traits observed among families made it difficult to estimate the QTL (Martinez *et al.* 2000).

Microsatellites are abundant simple sequences of 1–6 bp organized in tandem repeat arrays of variable number evenly distributed throughout the genome (Zane *et al.* 2002). Single-nucleotide polymorphisms (SNPs) between orthologous DNA regions represent the most abundant class of variation among alleles. The frequency of SNP across the Atlantic salmon genome has been estimated to 1/614 bp (Hayes *et al.* 2007), similar to the frequency (1/516 bp) recorded in Atlantic cod (Hubert *et al.* 2010). The analysis of sets of 30 000 EST of European sea bass and gilthead sea bream revealed a SNP frequency of 1/837 and 1/1014 bp, respectively (Louro *et al.* 2010). However, the study of SNPs in three chromosomes of European sea bass recorded a much lower average SNP frequency (1/2145 bp) and an uneven SNP distribution over the chromosomes and between introns, exons and intergenic areas (Kuhl *et al.* 2011). Single-nucleotide substitutions and microsatellites often show a Mendelian inheritance pattern. Although within a transcription unit the majority of SNPs are expected to be located in the intron regions, cDNA (ESTs) derived from mature mRNAs have proved a rich source of microsatellites and SNPs. By the end of 2011 dbEST contained approximately 230 000, 110 000 and 86 000 ESTs of *Gadus morhua*, *Sparus aurata* and *Dicentrarchus labrax*, respectively, and almost 84 000 of flatfishes (Pleuronectiformes). Microsatellite markers are now available for all major European cultured fishes and are efficiently used for individual identification, parental analysis and broodstock management (Saillant *et al.* 2006; Loukovitis *et al.* 2011). A tight linkage has been established between microsatellites and SNPs with loci directly involved in development and growth, as there is evidence that they modulate gene expression, making these markers promising candidates for marker-assisted selection of advantageous polymorphisms (Table 2).

In gilthead sea bream, length polymorphisms of the GH gene in both introns I and III have been suggested as markers for growth traits (Almuly *et al.* 2000). Sequence analysis of intron I variants revealed that the variation in length is mainly due to differences in the number of 17- or 15-mer (saGHFIM; Almuly *et al.* 2000). In addition, two alleles of the dinucleotide microsat saGHpCA located in the promoter of GH gene in gilthead sea bream were significantly correlated with high growth rates (Almuly *et al.* 2005). The functional role of saGHFIM in the transcription levels of the reporter gene was studied in different cell lines transfected with constructs containing intronic sequences of different lengths (Almuly *et al.* 2008). Interestingly, shorter intron I sequences enhanced reporter gene expression, in agreement with the observation that farmed sea bream under higher selection pressure had a higher proportion of shorter intron I (Almuly *et al.* 2008). A recent study in a sea bream commercial farm screened five candidate genes (GH, IGF-1, PRL, MSTN-1, SL) for polymorphism using PCR-RFLP analysis to reveal a significant association between a SNP in intron 2 and weight and length of broodstock, juvenile and adult fish (Sanchez-Ramos *et al.* 2012). Similarly, an analysis targeting GH, somatolactin and IGF-I genes in European sea bass, located a series of mini- and microsatellite sequences present in wild and farmed populations (Quere *et al.* 2010). In all cases simple sequence repeats (SSR) were located in their proximal promoter and/or intronic sequences. Only three SSR were detected in the 5′ region and a composite and one microsatellite were identified along the GH sequence (Quere *et al.* 2010). At the same time, a QTL analysis for body weight performed in European sea bass identified a QTL for growth in linkage group 1 (Chatziplis *et al.* 2007). A later study by Massault *et al.* (2010) also identified a QTL in LG1 for morphological traits along with another five QTLs in other linkage groups and two QTLs for body weight.

Atlantic cod (*Gadus morhua*) is a cold-water species that has been an important fisheries resource for centuries and is in the early stages of domestication. Studies on polymorphism of the species were initially undertaken for the development of markers for the identification of the different fish stocks in the North Atlantic. One of the molecules that

Table 2 Summary of polymorphism identified in candidate growth genes of European marine fish species

Species	Gene	Type of polymorphism	Position	Linkage to growth phenotype	Author
Dicentrarchus labrax	MSTN	6 microsat			Piñera *et al.* (2006)
Gadus morhua	Hb	Alleles HbI*1, HbI*2		HbI*2/2 = higher capture success and earlier feeding than other genotypes	Salvanes and Hart (2000)
Gadus morhua	Hb	Alleles HbI*1, HbI*2		HbI*2/2 = better fitted to transport O_2 at low temperatures	Brix *et al.* (2004)
Pagellus bogaraveo	MSTN	12 microsat			Piñera *et al.* (2006)
Paralichthys olivaceus	GH	Length polymorphism probably 19-mer variations in length in intron I	Intron 1- exon 2- intron 2	Differentiation in BW	Kang *et al.* (2002)
Sparus aurata	MSTN	7 microsat			Piñera *et al.* (2006)
Sparus aurata	MSTN1	RFLP allele (SNP)	Intron 2	Weight, length in broodstock, juvenile and adults	Sanchez-Ramos *et al.* (2012)
Sparus aurata	GH	Minisat 17-mer variations in length (VNTR)	Intron I	Weight, length	Almuly *et al.* (2000) and Sanchez-Ramos *et al.* (2005)
Sparus aurata	GH	Minisat 22-mer variations in length (VNTR)	Intron III		Almuly *et al.* (2000)
Sparus aurata	GH	Microsat $(CA)_{14}$ (saGHpCA)	Promoter	Weight	Almuly *et al.* (2005)
Sparus aurata	GH	−900 and −1700 bp upstream	Promoter	Resulted in lower luciferase activities	Almuly *et al.* (2008)
Sparus aurata	GH	Minisat 17-mer variations in length (VNTR)	Intron I	Shorter introns resulted in higher luciferase activities	Almuly *et al.* (2008)
Umbrina cirrosa	Myostatin (MSTN)	Microsat	3 UTR		Maccatrozzo *et al.* (2002)

early attracted the researchers' attention was haemoglobin, since there are two structures of this molecule as a result of polymorphism at the HbI* locus and an individual can be homozygous (HbI*1/1, HbI*2/2) or heterozygous (HbI*1/2). HbI*2/2 individuals exhibit faster growth rates and an earlier age of first spawning (Mork & Sundnes 1984; Portner *et al.* 2001), higher capture success (Salvanes & Hart 2000) and more efficient O_2 transport at low temperatures (Brix *et al.* 2004). The special performance of the HbI*2/2 genotype in cold waters was later attributed to non-synonymous mutations in the *Hb-β1* gene, resulting in the replacements Met55Val and Lys62Ala that differentiate the polar characteristics of the haem pocket regulating oxygen binding (Andersen *et al.* 2009).

The complement of expressed genes is tightly regulated both at transcriptional and post-transcriptional levels. Micro RNAs (miRNA) are a class of noncoding RNAs of about 22 nt that regulate gene expression at the post-transcriptional level by binding to the 3′ untranslated region (3′ UTR) of mRNAs and inducing their degradation or inhibiting their translation (Wienholds & Plasterk 2005). Several miRNAs are known to regulate muscle growth; for example, *miR-1* and *miR-206* promote satellite cell differentiation in mice by restricting their proliferative potential through downregulation of Pax7 (Chen *et al.* 2010). In zebrafish 168 miRNAs were expressed in the fast myotomal muscle over the whole life-cycle, including *miR-1, miR-133* and *miR-206* known to interact with transcriptional networks involved in myogenesis, while the regulation of expression of several miRNAs was associated with the transition from hyperplastic to hypertrophic growth during development (Johnston *et al.* 2009).

Genome mapping in aquaculture species has become possible through genetic and physical maps. Genetic maps arrange the genomic components in a possible order and suggest the linkage between them. Physical maps on the other hand, come in increasing level of detail. Increased research efforts and ample funding at a European level have made available a series of genomic resources for European sea bass; a >12× coverage BAC library (Whitaker *et al.* 2006), microsatellites (Johnston *et al.* 2009), combined linkage map (Chistiakov *et al.* 2005) were recently complemented by a radiation hybrid (RH) panel of 1581 ESTs and microsatellite markers (Guyon *et al.* 2010), comparative BAC mapping and low coverage shotgun sequencing (Kuhl *et al.* 2010). Using reference sequences from three assembled chromosomes and mapping all WGS data on them, a total of 20 779 SNPs were already identified over the 1469 gene loci and the intergenic space analysed (Kuhl *et al.* 2011). The EU Network of Excellence Marine Genomics

Europe developed EST projects for both sea bream and sea bass using 14 normalized tissue-specific cDNA libraries (Louro *et al.* 2010). Currently, the ESTs produced during those projects represent almost 50% of the species entries in NCBI dbEST and have been a valuable source for the mining of microsatellite markers and, to a lesser extent, of SNPs (Louro *et al.* 2010). Recently, using Next Generation Sequencing the transcriptome for white muscle in the sea bream was determined for adults and juveniles exposed to different nutritional states and temperature stress (Garcia De La Serrana *et al.* 2012). The annotated isotigs contained 5655 unique genes including 785 full-length cDNAs which mapped to 344 KEGG pathway maps. All major proteins of the sarcomere were present in the transcriptome (Fig. 3).

In the Atlantic cod, genomic resources for marker development from individuals enrolled in two current selective breeding programmes are being generated through an integrated genomics and broodstock development programme designed to be applied directly in two family-based breeding programmes in Canada (Bowman *et al.* 2011). More than 200 microsatellites have been identified and some have been used in extensive population analysis studies and the construction of linkage maps. At least 3000 potential SNPs have been identified from EST libraries in Canada and Norway and as many as 79 distinct miRNA species from collective RNA sample including 17 development stages from zygote to larval. In addition, a cod BAC library was recently generated for screening and isolation of particular genes (Shewring *et al.* 2011), which will offer new perspectives for functional genomics studies in this species.

The generation of genomic resources for flatfishes with a European dimension has lagged behind other aquaculture species (Cerda *et al.* 2010). Within the Pleuronectiformes, the most ESTs have been generated for halibut (*Hippoglossus hippoglossus*; Bai *et al.* 2007; Douglas *et al.* 2007). The ESTs have been a valuable source of microsatellite markers for halibut with 129 microsatellites identified, 60 of which were polymorphic (Douglas *et al.* 2007). A total of 258 microsatellite and 346 AFLP markers were incorporated into a genetic linkage map of 24 linkage groups consistent with the chromosomal number (Reid *et al.* 2007). Expressed sequence tag projects were also developed for highly prized Senegalese sole (*Solea senegalensis*) and turbot (*Scophthalmus maximus*) and formed the basis for the characteriza-

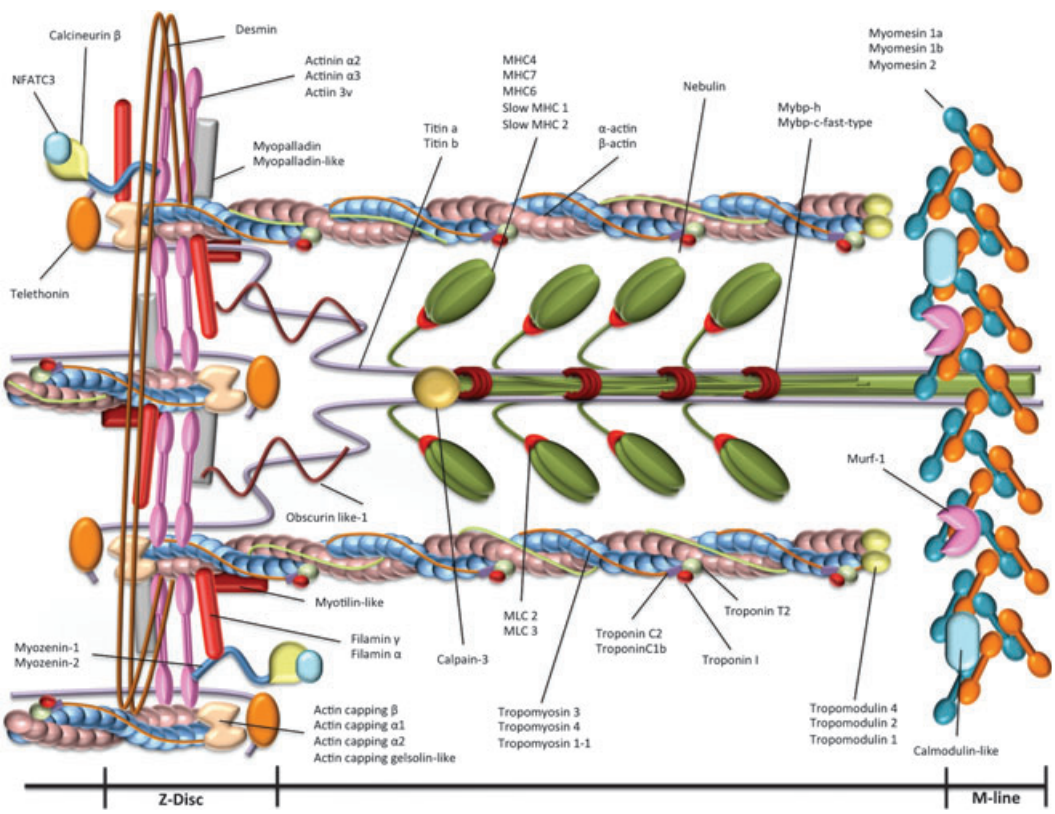

Figure 3 Sarcomeric proteins genes represented in the fast muscle transcriptome of the sea bream *Sparus aurata*. From Garcia De La Serrana *et al.* (2012).

tion of microsatellites (Bouza *et al.* 2008), the design of an oligonucleotide microarray and the development of a publicly available bioinformatic platform (Cerda *et al.* 2008).

Environmental factors and growth

Seawater temperature

Numerous studies have investigated the effects of temperature on muscle growth and differentiation in larval and juvenile stages of important species for European aquaculture. Different experimental designs have been employed: (i) clutches of eggs are split and incubated at different temperatures until hatching or metamorphosis and then on-grown at either constant or ambient temperature and (ii) individuals are reared at either constant or variable temperatures through all stages of development. Embryonic temperature (ET) affects the relative timing of muscle development and protein expression in fish embryos and larvae with respect to other morphological landmarks such as somite stage and body length (Hall *et al.* 2003). As a consequence of changes in the timing of fin and fin muscle development in relation to larval length the maximum swimming speed of Atlantic herring larvae was 24% higher in larvae hatched at 12°C ET than 5°C ET until 22 mm TL, with potential impacts on prey capture success and escape behaviour (Johnston *et al.* 2001). Increased embryonic temperature prior to first feeding is known to reduce the embryonic period and to have a significant effect on white muscle growth dynamics throughout the larval stages of important farmed marine species such as sea bass (Ayala *et al.* 2001; Alami-Durante *et al.* 2006), halibut (Galloway *et al.* 1999b), sole (Campos *et al.* 2012a), cod (Galloway *et al.* 1998) and Atlantic salmon (Stickland *et al.* 1988; Macqueen *et al.* 2008). However, after transference to similar rearing conditions free-swimming larvae that experienced cold embryonic temperature usually show substantial catch-up compensatory growth (Ayala *et al.* 2001; Macqueen *et al.* 2008). The impact of embryonic temperature on skeletal muscle cellularity and growth shows intra- and interspecific variations. In Senegalese sole, an embryonic temperature of 18 or 21°C produced larger larvae with 11% and 9% more fibres, respectively, after metamorphosis at equivalent ontogeny stages than those incubated at 15°C (Campos *et al.* 2012a; Dionísio *et al.* 2012). Nevertheless, the highest temperatures increased the incidence of skeletal deformities suggesting an experimental temperature regime of 18°C during egg incubation for achieving the best results regarding both growth and larval quality (Dionísio *et al.* 2012). Evidence for an optimal ET for life-cycle muscle fibre production was also obtained in Atlantic salmon where the final number of muscle fibres was highest at 5°C and was reduced at higher and lower treatment temperatures (Macqueen *et al.* 2008). Remarkably, the temperature during such a short window of embryogenesis dictated adult myogenic phenotype at later stages with significant treatment effects on the muscle fibre final number and size distribution in salmon and sea bass (López-Albors *et al.* 2008; Macqueen *et al.* 2008).

When individuals are reared at different temperatures through all stages of development, the muscle cellularity largely depends on the thermal conditions experienced by the embryo, the developmental stage considered and also the genetic origin of the fish (Gibson & Johnston 1995; Alami-Durante *et al.* 2007; López-Albors *et al.* 2008; Silva *et al.* 2011). White muscle hypertrophy was stimulated in embryos of European sea bass incubated at 20°C compared with 13 or 15°C, whereas in free swimming larvae the highest temperature stimulated both hyperplasia and hypertrophy (Alami-Durante *et al.* 2006). The same trend was observed in turbot (Gibson & Johnston 1995). Moreover, the hypertrophic and hyperplastic muscle growth of Mediterranean and Atlantic sea bass populations had different responses to temperature (Ayala *et al.* 2001). The selection of optimal incubation temperatures seems to have the most important impact on skeletal muscle growth a trait with major relevance to the aquaculture sector.

Nutrition

Nutrient availability is one of the most important factors influencing the growth performance of fish. Growth is protein deposition that is mainly regulated by the GH/IGF system. The effect of fasting and malnutrition on the GH–IGF axis is related to the absence of specific nutrients, or indirectly to nutritionally induced changes in hormonal status (Pérez-Sánchez & Le Bail 1999). Protein metabolism in fish larvae has been shown to be influenced strongly by the dietary amino acid profile, protein (or other forms of dietary nitrogen) digestibility, as well as dietary lipids. Diets promoting a fast growth rate were associated with a higher contribution of hyperplasia in the axial muscle of cod (Galloway *et al.* 1999a) and pike perch (*Sander lucioperca*) larvae (Ostaszewska *et al.* 2008) which in turn, promoted an increase in the body size of adult fish. Growth performances of fish larvae are sub-optimal and nitrogen retention low when diets are imbalanced in amino acid (AA) profiles (Aragão *et al.* 2004). Amino acid imbalances may also cause higher mortalities (Felip *et al.* 2012), and skeletal deformities (Pozios *et al.* 2001) in fish larvae. As skeletal deformities is a major problem in the marine hatcheries of Europe, amino acid imbalances in larval diets tend to have a negative impact on growth potential, and the development of a normal phenotype. In the case of nitrogen retention a higher variation was also observed in fish fed the imbalanced diet (Aragão *et al.* 2004), suggesting that individual variation in protein accretion response to dietary

stimuli needs to be taken into account. Several studies have also demonstrated that protein digestibility largely determines protein retention and growth. In particular, complex proteins, such as those present in fishmeal, are poorly digested, while free amino acids, peptides and hydrolysed proteins are better utilized by larvae (Conceição *et al.* 2011).

Excessive dietary lipid formulations may also have consequences in terms of protein utilization. Morais *et al.* (2005) observed that a diet with a higher lipid content affected gut morphology, amino acid metabolism and led to a lower growth rate. In juvenile Senegalese sole, the expression of the myogenic regulatory factors *mrf4*, *myod1*, *myod2* and *myog* was decreased in fast muscle with an increase in dietary lipid levels, resulting in reduced growth. Moreover, the *mlc2* transcript levels were highly correlated with protein ($r = 0.89$, $P < 0.05$) and lipid ($r = 0.82$, $P < 0.05$) gain (Campos *et al.* 2010). The effects of vitamin and essential fatty acids (EFA) deficiencies on fish larvae growth performance are poorly studied. In fact severe deficiencies in these nutrients are often documented to cause large larval mortalities (Hamre *et al.* 2010). However, the effect of milder deficiencies is less well studied, but it seems that EFA deficiencies might lead to growth depression. These effects of EFAs on survival and growth appear more pronounced in fish species with faster growing larvae (Hamre *et al.* 2010) with DHA exhibiting stronger effects compared with EPA. Moreover, EFA levels may interact with vitamin levels in defining optimal growth, and excessive EFA levels may cause growth depression and muscle lesions. European sea bass fed diets containing high DHA levels had improved growth, providing that the diet contained high vitamin E levels. However, excessive dietary DHA combined with low vitamin E levels led to an increased incidence of muscular lesions and poorer growth (Betancor *et al.* 2011). The effects of vitamins are less well studied, but Merchie *et al.* (1995) showed that very high ascorbic acid levels increased the larval growth rate at least in some fish species. The growth of haddock juveniles fed vitamin K deficient diets was depressed in the long term (Roy & Lall 2007).

Clearly a lot remains to be investigated about the extent to which optimal nutrition may contribute to meeting the growth potential of fish larvae. The recent identification and understanding of some of the molecular mechanisms underlying the nutritional regulation of protein accretion in muscle at early stages (described above) has an immense potential for improving feed efficiency in farmed fish.

Available methodology to assess growth and quality

The growth potential of fish larvae is shaped to a large extent by environmental and rearing conditions. Hence, the development of tools for the early prediction of larval performance and juvenile quality is crucial for the success of the aquaculture industry. The transcriptome, i.e. the entire collection of all transcripts in a species, is the link between information encoded in DNA and the phenotype. The tools for profiling different levels of the transcriptome have changed considerably over the years, from Northern blots and RT-PCR to expressed sequence tags (ESTs) and serial analysis of gene expression (SAGE). The development of gene expression microarrays has made possible the rapid and high-throughput quantification of the transcriptome along with the more recent advent of techniques for direct sequencing of the transcriptional output of the genome (RNA-seq). Integrating traditional approaches, such as histology, histochemistry and immunohistochemistry, with modern -omics techniques will improve our understanding of larval phenotype and its epigenetic regulation, which will enable the development of molecular markers of juvenile quality and the successful implementation of selective breeding programmes.

Histology, histochemistry and immunohistochemistry

Muscle cellularity (i.e. the number, diameter and type of fibres, their distribution, and density of myogenic progenitor cells) is directly related to growth and flesh quality of several farmed species (Johnston 1999; Valente *et al.* 1999, 2011; Periago *et al.* 2005). Staining muscle sections with haematoxylin and eosin is a rapid staining method used routinely to observe muscle fibres, whereas digital reproduction of the slides helps to determine the cellularity parameters. Fibre diameters computed with image analysis software can then be fitted to a smoothed probability density curve using a Kernel function, as described (Johnston *et al.* 1999). Muscle histology techniques have been used extensively to determine the growth dynamics of fast muscle fibres in several aquaculture species (Table 1), as well as the influence of environmental and nutritional factors on myogenesis.

Compared with haematoxylin and eosin staining, myofibrillar ATPase and succinate dehydrogenase histochemistry have the advantage of distinguishing different muscle fibre types based on their metabolic and contractile characteristics and was applied in several fish species (Martinez *et al.* 2000; Silva *et al.* 2008; Albors *et al.* 2010; Roy *et al.* 2012). The identification of particular proteins or their isoforms in muscle sections can be achieved by immunohistochemistry techniques. There are quite a few detection options although the biotin–streptavidin–peroxidase colorimetric method is the most commonly used (Rowlerson *et al.* 1997). Primary antibodies can detect myosin isoforms and many of them show cross-reactivity with a wide variety of fish species. For example, S58 is an IgA monoclonal antibody specific for slow isotypes of myosin heavy chain

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(*myhc*) in chicken but it recognizes slow-twitch muscle fibres in fish species such as the tiger pufferfish (Fernandes *et al.* 2005) and bluefin tuna (Roy *et al.* 2012). Polyclonal antibodies for various isoforms of myosin have given useful discrimination of fibre types in Sparidae (Rowlerson *et al.* 1997; Silva *et al.* 2008) and sole muscle fibres (Veggetti *et al.* 1999). Teleosts have a wide repertoire of myosin genes and some of these isoforms are regulated developmentally, have a fibre-specific pattern of expression (Steinbacher *et al.* 2006) and are differentially expressed with environmental conditions (Liang *et al.* 2007), suggesting that they may be suitable markers of growth. The density of myogenic progenitor cells can be a good indicator of growth potential, since changes in the number of muscle fibres possibly occur through stimulation of myoblast proliferation and/or delaying their differentiation (Brodeur *et al.* 2003). Fish muscle progenitor cells can be identified by immunohistochemistry using Pax3 and Pax7 as markers (Stellabotte *et al.* 2007; Marschallinger *et al.* 2009).

In situ hybridization

In situ hybridization is used to localize specific mRNA or miRNA transcripts in tissue sections or whole embryos (whole-mount *in situ*) by hybridizing a labelled probe to its complementary target. This technique has been used widely to determine the developmental expression pattern of several growth-related related genes (Barresi *et al.* 2001; Baxendale *et al.* 2004; Steinbacher *et al.* 2006). In tiger pufferfish, the developmental plasticity of the expression of *myogenin* and the forkhead/winged helix transcription factor *foxk1* and its splice variants was determined using digoxigenin-labelled probes, an alkaline phosphatase-conjugated anti-digoxigenin antibody and 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue tetrazolium for colorimetric detection (Fernandes *et al.* 2006, 2007). Several studies have used *in situ* hybridization to study the expression of myosin heavy chains (Cole *et al.* 2004; Johnston *et al.* 2009) or myosin light chains (Moutou *et al.* 2005; Silva *et al.* 2010) specific to myotubes and immature muscle fibres, which can be used as markers for hyperplastic growth.

miRNAs can also be detected by *in situ* hybridization with locked nucleic acid (LNA)-modified DNA probes (Kloosterman *et al.* 2006). This method has been used to determine the temporal and spatial expression of 115 conserved vertebrate miRNAs in zebrafish embryos, revealing that most miRNAs play a role in tissue differentiation, but not in fate establishment (Wienholds *et al.* 2005).

Real-time PCR

Amongst the various methods available to quantify gene expression, fluorescence-based real-time PCR (qPCR) with SYBR green chemistry or TaqMan probes is still the method of choice, due to its high sensitivity and accuracy. Nevertheless, in order to obtain sensible and reproducible results it is essential to be aware of qPCR pitfalls, which have been summarized in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009). In particular, the reference genes used for standardization are critical, since most quantitative data are relative, not absolute. Reference genes have been validated for several aquaculture species, including Atlantic halibut (Fernandes *et al.* 2008), Senegalese sole (Infante *et al.* 2008), Atlantic salmon (Olsvik *et al.* 2005), European sea bass (Mitter *et al.* 2009) and Atlantic cod (Nagasawa *et al.* 2012). The data from these publications should be interpreted as recommendations, since there is no such thing as a universal reference gene. It should be an integral part of any qPCR study to validate the reference genes for a particular biological context. This is especially important when quantifying expression throughout embryonic development, as many commonly used reference genes show a dynamic expression pattern and are unsuitable to standardize target gene profiles during early ontogeny (Campos *et al.* 2012a,b). In such instances, it may be better to use an exogenous reference gene (e.g. firefly luciferase) for data normalization.

The applications of qPCR go beyond simple quantification of gene expression. In particular, the relatively novel high resolution melting (HRM) analysis, which is based on the dissociation behaviour of qPCR products, enables the discrimination of samples based on their sequence and single base differences can be detected. This can be applied to the investigation of SNPs that may affect growth potential. When combined with sodium bisulphite treatment of DNA, HRM can also be used to quantify methylation, thus becoming a valuable tool for epigenetic studies.

Microarrays

Microarrays are used to examine differential expression of hundreds or even tens of thousands of genes simultaneously. In addition to this obvious advantage, this type of analysis elucidates relationships between known genes and genetic pathways. Each microarray contains a large number of cDNA or oligonucleotide spots that hybridize with fluorescently labelled probes. There are a number of factors that need to be considered when planning a microarray experiment, namely the experimental design, platform and probe labelling (reviewed by Stears *et al.* 2003). The large number of experiments and projects based on microarrays has produced a range of mature strategies for experimental design and subsequent data analysis (Aragão *et al.* 2004).

With the decrease in DNA sequencing costs there has been a dramatic increase in genomic resources for aquaculture

species in the past decade. In particular, a large number of ESTs has been generated from genome and transcriptome sequencing projects (Canario *et al.* 2008). The availability of these molecular tools has enabled the construction of several microarray platforms for commercially important fish species (Table 3). These microarrays have been used in a number of transcriptomic studies pertaining to aquaculture issues, including the Atlantic cod immune response (Booman *et al.* 2011), metamorphosis in Atlantic halibut (Douglas *et al.* 2008), jaw deformities in European sea bass (Ferraresso *et al.* 2010) and the stress response in gilthead sea bream (Sarropoulou *et al.* 2005; Calduch-Giner *et al.* 2010).

Transcriptome analysis and genome editing

Suppression subtractive hybridization (SSH) and serial analysis of gene expression (SAGE) are powerful techniques to compare mRNA transcripts between two samples. For example, by comparing fast muscle transcriptomes from juvenile fish that were still growing by hyperplasia with adult fish that had stopped recruiting, Fernandes *et al.* (2005) have used SSH to discover novel genes that may be involved in myotube formation in the tiger pufferfish (*Takifugu rubripes*). More recently, this approach has been used to identify nutritionally regulated genes involved in the growth of fast skeletal muscle in Atlantic salmon by comparing libraries from fish with zero growth rates to fish growing rapidly (Bower & Johnston 2010).

The main advantage of SSH and SAGE over microarray analyses is that one is not restricted to a pre-defined number of probes spotted on the array. However, the preparation and characterization of SSH or SAGE libraries is labour intensive and if using traditional sequencing methods, such as Sanger sequencing, the number of cDNA clones analysed is also relatively limited. RNA-Seq is a revolutionary technology for the accurate comparison of transcriptome profiles based on next-generation sequencing (Wang *et al.* 2009). This method is based on the repeated sequencing of a DNA fragment in a very short time ensuring increased sensitivity and accuracy. Transcript sequences are then mapped back to a reference genome and the counts of each read are then used to assess the level of gene expression based on the assumption that the number of mapped reads reflects the expression level for that gene or genomic region. Compared with microarrays, RNA-Seq provides direct access to the sequence, junctions between exons can be assayed without prior knowledge of the gene structure, RNA editing events can be detected, and knowledge of polymorphisms can provide direct measurements of allele-specific expression. Finally, because RNA-Seq provides direct access to the sequence it can be used on species for which a full genome sequence is not available, whereas the only option in this case for microarrays is to hybridize RNA to a microarray designed for another species, which has limitations because of sequence divergence. Another strength of RNA-Seq is in the quantification of individual transcript isoforms. Alternative splicing, although acknowledged as an important source of functional diversity in eukaryotes, has been relatively little studied at the level of the transcriptome, principally because of the difficulty of measuring expression for each isoform. Analyses of RNA-Seq reads that span exon/exon boundaries make it possible to identify and compare diversity and abundance of gene isoforms. One of the few published studies applied this technique to unveil the genetic basis for the phenotypic diversity between siscowet and lean lake trout (*Salvelinus namaycush*), which differ in growth and lipid content (Goetz *et al.* 2010).

Table 3 Examples of available microarrays for commercially important fish species

Species	Array type/size	Unique probes	Author
Atlantic cod (*Gadus morhua*)	Oligo	20 000	Booman *et al.* (2011)
	cDNA	16 348	Edvardsen *et al.* (2011)
Atlantic halibut (*Hippoglossus hippoglossus*)	Oligo/44K	9277	Douglas *et al.* (2008)
Atlantic salmon (*Salmo salar*)	Oligo/44K	21 323	Krasnov *et al.* (2011)
	Oligo/44K	32 527	Jantzen *et al.* (2011)
Common carp (*Cyprinus carpio*)	cDNA	13 440	Gracey *et al.* (2004)
European sea bass (*Dicentrarchus labrax*)	Oligo/44K	19 048	Ferraresso *et al.* (2010)
Gilthead sea bream (*Sparus aurata*)	cDNA	10 176	Sarropoulou *et al.* (2005)
	Oligo/44K	19 715	Ferraresso *et al.* (2008)
	cDNA/	18 490	Calduch-Giner *et al.* (2010)
Rainbow trout (*Oncorhynchus mykiss*)	cDNA	16 006	Salem *et al.* (2006)
	cDNA	9023	Rescan *et al.* (2007)
	Oligo	37 394	Salem *et al.* (2008)
Senegalese sole (*Solea senegalensis*)	Oligo	5087	Cerda *et al.* (2008)

Illumina, 454, SOLiD and ion-torrent next-generation sequencing technologies represent an efficient and cost-effective way of obtaining transcriptome data for non-model organisms (Metzker 2010) and it is likely that they will be applied widely in the near future to investigate growth potential in aquaculture species. In addition to mRNA profiling, they have been used to determine SNPs (Kuhl *et al.* 2011) and to examine changes in the small RNA transcriptome (Bizuayehu *et al.* 2012). Next-generation sequencing is also a promising tool to analyse global DNA methylation patterns that may help in understanding the phenotypic plasticity of growth observed in teleosts. Zinc finger nucleases are powerful tools for genome editing, since they can be custom-designed to cut at specific DNA sequences (Carroll 2011; Wood *et al.* 2011). By combining the non-specific cleavage domain of FokI endonuclease with a DNA-binding domain of zinc fingers, site-specific DNA breaks can be created. The repair of these breaks then results in insertions and deletions that disrupt the reading frame of the targeted gene. This technique has been applied recently to knock-out one myostatin (*mstn*) paralogue in the yellow catfish, *Pelteobagrus fulvidraco* (Dong *et al.* 2011). The mutation was inheritable and the F1 yellow catfish strain carrying the null *mstn* allele will certainly be useful to explore the roles of *mstn* in fish growth or even to produce yellow catfish with a higher muscle mass.

Another type of artificial restriction enzymes that have recently emerged as molecular scissors for targeted gene disruption are the transcription activator-like effector nucleases (TALENs). They are engineered by fusing a type II FokI DNA cleavage domain with a transcription activator-like domain, which contains a number of amino acid repeats that recognize specific nucleotides (Wood *et al.* 2011). The application of TALENs to editing fish genomes is still in its infancy, but Cade *et al.* (2012) have demonstrated that TALENs induce high rates of heritable mutations in eight endogenous zebrafish genes, namely *gria3a*, *hey2*, *elmo1*, *epas1b*, *fh*, *hif1ab*, *ptpmt1* and *slc6a3*.

Proteomics

Protein expression allows the assessment of functional and/or structural effects caused by nutritional and/or environmental conditions. Two-dimensional electrophoresis, which combines the separation of proteins according both to their isoelectric point (by isoelectric focusing) and molecular weight (by SDS-PAGE; Lopez 2007), followed by identification of the proteins of interest by mass spectrometry (Canas *et al.* 2006) is the traditional method used for proteome analysis. Nowadays, more sensitive methodologies are used increasingly, including differential in-gel electrophoresis (DIGE; Link *et al.* 2006) and iTRAQ labelling and protein separation by LC coupled with MS/MS (Wiese 2007; Martyniuk & Denslow 2009). Functional proteomics methodologies are complementary to microarray and mass sequencing techniques, in expanding the focus from target genes/proteins to unbiased analyses of the genome/proteome. Proteomics may also provide information on post-translational modifications (e.g. glycosylation, phosphorylation, acetylation, ubiquitination).

The few proteomic studies conducted so far on fish larvae have focused on changes of proteome expression during fish development (Focant *et al.* 2003; Link *et al.* 2006; Sveinsdottir *et al.* 2008; Gomez-Requeni *et al.* 2010), or dietary effects (Gomez-Requeni *et al.* 2011). Despite methodological difficulties (Conceição *et al.* 2010) related to sample size and poor annotation of the genome of many marine species, these studies have pointed out important clusters of regulated proteins, some of which are related to muscle accretion.

Cell culture

Cell culture and the development of stable cell lines provide important biological tools for carrying out investigations in physiology, development and gene regulation. A recent review (Lakra *et al.* 2011) reported the existence of 283 finfish cell lines. Primary muscle cell culture of rainbow trout has been a valuable tool in studying the regulation of metabolic pathways by growth factors and the role of different signalling pathways (Codina *et al.* 2008; Cleveland & Weber 2010) that has been adopted successfully for relevant studies in gilthead sea bream to show that besides stimulating myoblast proliferation, IGF-I and IGF-II also induce differentiation, through upregulation of myogenin (Montserrat *et al.* 2007).

At the same time, isolated adipocytes of Atlantic salmon and rainbow trout at different stages of differentiation have been used to investigate the differentiating gene expression profiles in adipocytes of different origin (Weil *et al.* 2009), the sequence of gene regulation and lipid metabolism events during differentiation and maturation (Bouraoui *et al.* 2008), the relationship between lipid storage and immune responses in white adipocytes (Todorcevic *et al.* 2010). Isolated adipocytes from gilthead sea bream have also been used to examine the effects of diet composition (fish meal vs. plant proteins) and fasting on lipolysis, the effects of insulin, glucagon and GH in adipocytes isolated from fish with different nutritional histories (Albalat *et al.* 2005) as well as to explore the heterogeneity of adiposity based on the response to TNFa and its receptors (Cruz-Garcia *et al.* 2009).

Overall, fish primary cell cultures can form the basis for the development of functional assays of novel genes (over-expression/knock-down) in order to elucidate their roles in the development and differentiation of cell types

contributing to body mass growth and for the investigation of the effects of nutritional or environmental factors on muscle and bone progenitor cell proliferation and differentiation. This knowledge at the cellular level can provide basic metabolic and gene networks and act as a catalyst for the synthesis of the systemic function to be verified through whole-organism experiments. The development of co-cultures of different cell types will be important in gaining a better understanding of crucial cell–cell interactions that might determine the cell fate and foster this holistic view.

Tracer studies

Considerable progress has been reported on the use of tracer methodologies in fish larval research (Conceição *et al.* 2008, 2010). Some of these techniques may be instrumental to improve the understanding of growth potential as affected by nutritional and environmental factors. Tube feeding of a radiolabelled nutrient (normally ^{14}C-labelled), followed by quantification of the tracer that is present in faeces, retained in tissues and catabolized, after some hours, has been used to assess protein retention and also the digestion/absorption capacity for amino acids, peptides and proteins (Conceição *et al.* 2008, 2010). Incorporation into *Artemia* of ^{14}C-amino acids has also been used to study *Artemia* protein retention in fish larvae (Morais *et al.* 2004; Engrola *et al.* 2009, 2010).

Tracer studies may also be used to measure the rates of protein synthesis and protein turnover in fish larvae (Conceição *et al.* 2008, 2010). A tracer, e.g. L-[2,6-^{3}H]phenylalanine or a mix of ^{15}N-amino acids, is supplied via an immersion bath (Houlihan *et al.* 1995a; Conceição *et al.* 1997a,b) or the feed (Conceição *et al.* 2001). Rates of protein synthesis and turnover are sensitive indicators of nutritional condition (Houlihan *et al.* 1995a; Conceição *et al.* 1997b), ontogenetic growth potential (Conceição *et al.* 1997a) and immunostimulation (Conceição *et al.* 2001).

Even if the results obtained using tracer studies are short-term, and do not necessarily represent the performance of larvae in the long term, they can be useful tools for assessing the protein retention phenotype and for comparing performance between treatments, as well as ontogenetic changes (Conceição *et al.* 2010).

Concluding remarks

The future growth and competitiveness of the European aquaculture industry depends on an increased scientific knowledge of the biology of muscle growth, the genetic basis of flesh quality traits and the influence of environmental factors on growth and product quality. Research will need to focus on the identification of crucial windows in development that introduce maximum growth variation; the development of high-throughput methodologies for screening larvae as early as possible for growth potential; the use of genetic resources to identify candidate genes and to study their polymorphisms; the interactions between nutritional/environmental factors and gene expression, and their effect on amino acid fluxes and protein deposition; the incorporation of novel genetic resources in marker-assisted selection programmes. Advances in technology and the reduced cost of sequencing will enable detailed genetic information to be obtained for every aquaculture species. The resulting dramatic interest in sequence data will enable marker assisted selection for superior larval survival, enhanced muscle growth and disease resistance to become the norm within 5–10 years. In addition to the muscle fibres, skeletal muscle contains many cell types including adipocytes, fibroblasts, osteocytes, capillary endothelial cells, macrophages and leucocytes. Interactions between these components are poorly understood but undoubtedly play a significant role in determining growth and juvenile quality.

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